

117-231

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. Application No. (If known, see 37 C.F.R. 1.5)

08776,350

International Application No.

PCT/GB95/01791

International Filing Date

28 July 1995

Priority Date Claimed

29 July 1994

Title of Invention

TREATMENT OF CANCER USING HSV MUTANT

Applicant(s) For DO/EO/US

MACLEAN et al

Applicant herewith submits to the United States Designed/Elected Office (DO/EO/US) the following items and other information:

1. ☐ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☒ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371 (c)(2)).
7. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau
 - c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).
11. The above checked items are being transmitted:
 - a. ☐ before the 18th month publication.
 - b. ☐ after publication and the Article 20 communication but before 20 months from the priority date.
 - c. ☐ after 20 months.
 - d. ☐ by 30 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
 - e. ☐ after 30 months.

Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted (1) after 20 months and no proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date, or (2) after 30 months and a proper demand for International preliminary Examination was made by 19 months from the earliest claimed priority date.
12. At the time of transmittal, the time limit for amending claims under Article 19
 - a. ☐ has expired and no amendments were made.
 - b. ☐ has not yet expired.
13. ☒ Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on January 28, 1997, namely:
Application papers, Preliminary Amendment, Information Disclosure Statement, International Search Report and filing fees

Items 14. to 19. below concern other document(s) or information included:

14. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
15. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
16. ☐ A **FIRST** preliminary amendment.
- ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.

NO DATA PCT-TO 23 JAN 1997

117-231

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18. ☐ A change of power of attorney and/or address letter.

19. <input checked="" type="checkbox"/> Other items or information:					
International Search Report.					
20. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): -- Search Report has been prepared by the EPO or JPO \$910.00 -- International preliminary examination fee paid to USPTO (37 CFR 1.492)..... \$700.00 -- No international preliminary examination fee paid to USPTO (37 CFR 1.492) but international search fee paid to USPTO (37 CFR 1.445 (a)(2))..... \$770.00 -- Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$1,040.00 -- International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provision of PCT Article 33(1) to (4) \$96.00				CALCULATIONS	PTO USE ONLY
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$ 910.00	
Surcharge of \$130.00 for furnishing the National fee or oath or declaration later than					
[] 20 [<input checked="" type="checkbox"/>] 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	22 - 20 =	2	X \$ 22.00	\$ 44.00	
Independent Claims	2 - 3 =	0	X \$ 80.00	\$ 0.00	
Multiple Dependent Claim(s) (if applicable)			+ \$260.00		
TOTAL OF ABOVE CALCULATIONS =				\$ 1,084.00	
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28).				\$	
SUBTOTAL =				\$ 1,084.00	
Processing fee of \$130.00, for furnishing the English Translation later than					
[] 20 [] 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
TOTAL NATIONAL FEE =				\$ 1,084.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$	
Fee for Petition to Revive Unintentionally Abandoned Application (\$1,290 -- Small Entity Fee = \$645)				\$	
TOTAL FEES ENCLOSED =				\$ 1,084.00	
				Amount to be refunded	\$
				Charged	\$
a. <input checked="" type="checkbox"/> A check in the amount of.....\$ <u>1,084.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 14-1140 in the amount of \$ _____ to cover the above fees. A duplicate copy of this form is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>14-1140</u> . A duplicate copy of this form is enclosed.					
SEND ALL CORRESPONDENCE TO:				Signature	
NIXON & VANDERHYE P.C. 1100 North Glebe Road, 8th Floor Arlington, Virginia 22201 Telephone: 703-816-4000				Arthur R. Crawford	
				Name	
				25,327	January 28, 1997
				Registration Number	Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

MACLEAN et al
(National Phase PCT/GB95/01791)

Atty. Ref.: 117-231

Serial No. (To Be Assigned)

Group:

Filed: 28 January 1997

Examiner:

For: TREATMENT OF CANCER USING HSV MUTANT

January 28, 1997

Honorable Commissioner of Patents
and Trademarks
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

In order to place the above-identified application in better condition for examination, please amend the above-identified application as follows:

IN THE CLAIMS:

Please delete Claims 1 through 19 without prejudice.

PLEASE ADD THE FOLLOWING NEW CLAIMS -- 20 THROUGH 42 --

-- 20. A method of treating a secondary metastatic tumor in the central nervous system of a mammal, which method comprises the step of administering to the said mammal an effective amount of a mutant herpes simplex virus type 1 which has a non-functional $\gamma 34.5$ gene in the long repeat region (R_L).

21. A method according to claim 20, wherein the mammal is a human.

22. A method according to claim 20, wherein the secondary metastatic tumor occurs in the brain.

23. A method according to claim 22, wherein the secondary tumor is a metastasised melanoma.

24. A method according to claim 20, wherein the mutant virus is a mutant strain 17 virus.

25. A method according to claim 20, wherein the mutant virus is a herpes simplex virus type 1 which has been modified within the Bam H1 s restriction fragment of the R_L terminal repeat.

26. A method according to claim 25, wherein the modification is a deletion of from 0.1 to 3kb.

27. A method according to claim 26, wherein the deletion is from 0.7 to 2.5 kb.

28. A method according to claim 27, wherein the deletion is a 759 bp deletion in the γ 34.5 gene.

29. A method according to claim 20, wherein the mutant virus is strain 1716.

30. A method of treating a cancer in a mammal, which method comprises the step of administering to the said mammal an effective amount of a mutant herpes simplex virus type 1 wherein the mutant virus is a mutant strain 17 virus which has a non-functional γ 34.5 gene in the long repeat region (R_L).

31. A method according to claim 30, wherein the mammal is a human.

32. A method according to claim 30, wherein the cancer is a brain tumor.

33. A method according to claim 32, wherein the tumor is a primary tumor originating within the brain or nervous system.

34. A method according to claim 30, wherein the cancer is a metastatic tumor.

35. A method according to claim 34, wherein the cancer is a metastasised melanoma.

36. A method according to claim 30, wherein the mutant strain 17 virus has been modified within the Bam H1 s restriction fragment of the R_L terminal repeat.

37. A method according to claim 36, wherein the modification is a deletion of from 0.1 to 3kb.

38. A method according to claim 37, wherein the deletion is from 0.7 to 2.5 kb.

39. A method according to claim 38, wherein the deletion is a 759 bp deletion in the γ 34.5 gene.

40. A method according to claim 30, wherein the mutant virus is strain 1716.

41. A method according to claim 30, wherein the mutant virus is administered directly into the cancer.

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42. A method according to claim 30, wherein the mutant virus is administered parenterally into the blood stream feeding the cancer. --

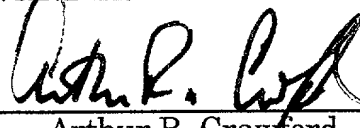
REMARKS

Favorable consideration of this application and entry of the foregoing amendments are respectfully requested.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____



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Rec'd PCT/PTO 28 JAN 1997

TREATMENT OF CANCER USING HSV MUTANTFIELD OF INVENTION

The present invention relates to the use of a herpes simplex virus (HSV) mutant for the treatment of cancer tumors, particularly those of the brain or nervous system whether the tumors are metastatic tumors or primary tumors.

BACKGROUND

The DNA sequence of herpes simplex type 1 (HSV-1) is known (references 13,25) and is linear with a length of about 152k residues. It consists of two covalently linked segments, designated long (L) and short (S). Each segment contains a unique sequence flanked by a pair of inverted terminal repeat sequences. The long repeat (R_L) and short repeat (R_S) are distinct. The unique long (U_L) region includes genes UL1 to UL56, and the U_S region includes genes US1 to US12.

A relatively large number of patients with advanced cancers will develop metastatic lesions in the brain and spinal cord. This frequently results in severe and debilitating neurological complications including headache, paralysis, seizures, and impaired cognition. It has been estimated that 70,000 cancer deaths occur each year in the United States with metastatic lesions to the central nervous system

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(CNS). Radiation and steroids are presently the principle therapies used, however, they are only palliative, and frequently cause significant neuropsychological and endocrinological morbidity. Surgery is generally reserved for removal of solitary metastases, and is often not curative (1).

Viral therapy for the destruction of tumors is not a new concept. Effects in various experimental tumor systems have been demonstrated using parvovirus H-1, Newcastle disease virus, retroviral vectors containing drug susceptibility genes, and Herpes Simplex Type I virus (HSV-1) (2-7). The mechanisms by which viruses improve the outcome in experimental tumor systems are complex and poorly understood. Brain tumors represent a dividing cell population occurring within an essentially non-dividing cell population of support cells, and terminally differentiated neurons. Thus, in the context of brain tumor therapy, one rationale is to select a virus that replicates exclusively or preferentially in dividing cells. Such a virus may be capable of establishing a lytic infection exclusively in tumor cells within the CNS, ultimately destroying the tumors without infecting surrounding brain, and without deleterious effects to the host.

Pioneering experiments with HSV showed a dose dependent improvement in survival of nude mice bearing intracranial human gliomas following intratumoral

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therapy with mutant HSV-1 dlspTK (3). This virus has a deletion in the viral thymidine kinase (TK) gene, (8) and exhibits a relatively neuro-attenuated phenotype in mice (9). However, dlspTK infection of tumor bearing animals causes histologically evident encephalitis (3). The use of TK mutants of HSV-1 for viral therapy also has an inherent major disadvantage in that these viruses are resistant to the clinically effective anti-viral agents acyclovir and ganciclovir (10).

The terminal 1 kb of the long repeat region (R_L) of the HSV-1 and HSV-2 genomes contain a gene (11-13), that confers neurovirulence. Deletion or mutation of this gene ($\gamma 34.5$), results in variants that grow as well as wild type virus on dividing cells of many established cell lines, but show impaired replication on non-dividing cells (12-14). In mice, $\gamma 34.5$ null mutants are incapable of replicating in the central nervous system, and do not cause encephalitis (12,15-16).

A mutant HSV-1 called R3616, containing a 1000 base pair (bp) deletion in $\gamma 34.5$, with an LD_{50} (minimum dose of virus that kills 50% of infected animals) that is at least 3×10^3 fold greater than wild type F strain virus from which it was derived (12), has been shown to improve the outcome of nude mice bearing intracranial human gliomas (17). In the work presented here, we have utilized an HSV-1 strain 17

mutant virus called 1716, that has a 759 bp deletion in γ 34.5 (16).

The construction of mutant virus 1716 is described in published patent application W092/13943 (PCT/GB92/00179) the contents of which are incorporated herein by reference. However, this patent publication is solely concerned with the use of mutant 1716 as a vaccine, either in itself or as a vector vaccine which includes a heterologous gene coding for an antigen.

Melanoma is a prevalent malignancy. Cerebral metastases occur in up to 75% of patients with metastatic disease, and are among the most common causes of death (18-22). Presently, the life span of patients with CNS melanoma is short, ranging from 2 to 7 months (23).

It is an object of the present invention to provide an improved HSV-based viral therapy of cancer tumors.

STATEMENT OF INVENTION

The present invention in one aspect provides the use as an anticancer agent of a mutant herpes simplex virus which has been modified in the γ 34.5 gene of the long repeat region (R_L) such that the gene is non-functional.

The invention also relates to a method of treatment of cancer in a mammal (human or animal) by

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the administration to the mammal of an anti-cancer effective dose of the mutant herpes simplex virus.

DETAILED DESCRIPTION

For the purposes of the present invention "non-functional" means that the gene has been modified by deletion, insertion or substitution (or other change in the DNA sequence such as by rearrangement) such that it does not express the normal product or a functionally equivalent product. The effect of the non-functionality of the gene is that the neurovirulence of the virus to the patient is substantially removed.

Thus the invention is based on the finding that rendering the γ 34.5 gene non-functional provides an HSV mutant which is particularly effective in destroying dividing tumor cells, whilst at the same time the HSV mutant does not replicate within normal non-cancerous cells. It therefore has the potential to provide a safe anti-cancer treatment.

Two types of herpes simplex virus are known HSV-1 and HSV-2 and either may be employed in the present invention to provide the HSV mutant. Inter-type recombinants containing DNA from both types could also be used.

The modification may be effected at any convenient point within the γ 34.5 gene, and such point generally corresponds to a restriction enzyme site.

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The modification may be within the Bam H1 s restriction fragment of the R_L terminal repeat (corresponding to 0-0.02 and 0.81 - 0.83 mu). The modification is typically a deletion of 0.1 to 3kb, particularly 0.7 to 2.5kb. In this work a 759 bp deletion in γ 34.5 was made in the HSV-1 mutant, referred to as 1716.

The HSV genome also includes a number of other genes which are non-essential to the successful culturing of the virus. It is, of course, necessary to retain within the HSV mutant the ability to culture the mutant so that the mutant is self-replicating and stocks of the mutant can be grown in tissue culture. Lethal modifications of the genome which remove the ability to culture the HSV mutant are not acceptable.

However, in addition to the primary modification to the γ 34.5 gene of the R_L region, it may be advantageous to also include in the HSV mutant one or more secondary non-lethal modifications within non-essential genes.

The present invention also encompasses as a new product an HSV mutant which includes in addition to the primary modification, a secondary non-lethal modification (for example within Vmw65). The mutant may be derived from HSV-1 or HSV-2.

In a similar way, other secondary modifications may involve modification of the latency associated transcript (LAT) promoter so as to render the promoter

non-functional and prevent transcription thereof.

Herpes simplex virus infects the brain and nervous system. The HSV mutant is effective against primary tumors originating within the brain and nervous system, but is particularly useful against metastatic tumors where cancer cells originating elsewhere have lodged in the brain or nervous system (particularly the central nervous system (CNS)). Brain metastases occur commonly in a variety of human cancers (e.g. melanomas), and at present such cases are invariably fatal. The efficacy of treatment according to the invention employing the HSV mutant will depend on the time after origination of the tumor at which the treatment is initiated, but efficacy is improved by early treatment for example in 1 to 30 days.

The LD₅₀ (minimum dose of virus that kills 50% of infected animals) of the 1716 mutant in respect of mice is 10⁶ fold greater than that of the wild type 17+ virus from which it is derived. Thus the neurovirulence of 1716 is essentially removed relative to the wild type virus.

The effective non-toxic dose of HSV mutant can be determined by routine investigation by the skilled addressee, and will depend on a number of factors including the particular species of mammal and the extent of development of the tumor. A guide can be obtained from the Examples herein.

In a further aspect of the invention there is

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provided a method of treating cancer in mammals, in particular in humans by administering a pharmaceutical formulation comprising the HSV mutant to mammals, in particular to humans. Thus, the method of treatment can comprise the administration of a pharmaceutical formulation comprising the HSV mutant by injection directly into the tumour or parenterally into the blood stream feeding the tumour.

It will usually be presented as a pharmaceutical formulation including a carrier or excipient, for example an injectable carrier such as saline or apyrogenic water. The formulation may be prepared by conventional means.

Embodiments of the invention will now be described by way of example only.

Figures 1 and 2 show the results of experiments described fully in Examples 3 and 5 respectively.

Figure 1: Survival Curves

Tumor-bearing mice injected at 10 days (Figure 1b) and at 5 days (Figure 1a) post tumor injection with HSV-1 mutant 1716.

Figure 2: Relative replication rates of HSV-1 mutant
Relative replication rates of HSV-1 mutant 1716 in brain tumor (closed squares); and of 1716 and wild type 17+ in non-tumor brain (open squares and closed triangles respectively).

Figure 3: HSV-1 Genome map.

HSV-1 genome showing approximate location of the

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γ 34.5, 2kb latency associated transcript (LAT) and neighbouring genes. A. The 152kb HSV-1 strain 17+ genome, illustrating the unique long and short segments of the genome, U_L and U_S (lines), bounded by internal (IR) and terminal repeat (TR) regions (open boxes). Hatch marks show location of the VP16, thymidine kinase (TK) and glycoprotein C (gC) genes. B. Expanded view of the U_L/U_S region of the genome the location of the γ 34.5, ICPO and ICP4 mRNAs and the location of the 2.0kb LAT which is expressed during acute and latent infection. C. The location of the 759 bp deletion in strain 1716. D. The location of the LAT specific BstEII-BstEII probe used for in situ detection of HSV specific gene expression. Nucleotide positions are based on DNA sequence analysis of Perry and McGeoch (45).

Figure 4: Quantification of infectious virus in nude mouse brain after IC inoculation.

To investigate the extent of strain 1716 replication in brain tumors, nude mice were injected with NT2 cells. Twelve days later each mouse was infected with 5×10^5 PFU of strain 1716 at the same stereotactic coordinates (open circles). At the times indicated, mice were sacrificed, the brains were frozen in Liquid N_2 and stored at $-70^\circ C$. Specimens were thawed rapidly, homogenized, and viral titration was performed in triplicate on BHK cells. To establish the growth characteristic of strain 1716 and parental 17+ in

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brain without tumor, mice were injected intracranially with either 5×10^4 PFU of 1716 (closed circles) or 1×10^6 PFU of 17* (closed triangles). Mice were sacrificed at the times shown and processed as described in Methods. Each point is the mean of 2 mice with SEM bars.

Figure 5: Detection of replicating virus by immunohistochemistry and in situ hybridization

Nude mice were IC injected with 3×10^4 NT2 cells. 14 days later they were inoculated with 5×10^5 PFU of 1716. A. Control mice with tumor after 14 days. B. Tumor is histologically very diverse, arrows: tubular structures. C. MOC-1 antibody specifically identifies the NT2 tumor cells. D. Antibody MIB-1 identifies cycling cells. Note low number of labeled cells in tubular structures (asterisk). E. 14 day tumor after three days infection with strain 1716. The arrow indicates a region of extensive tumor lysis and necrosis. F. Infected cells show characteristic features of herpes infected cells such as intranuclear inclusion bodies formation, cytomegaly and necrosis. G. Herpes antigen is limited to tumor cells. H. Virus replicates in tumor cells at interface between tumor and host brain shown using anti-HSV antibodies. I. The kinetics of viral replication are delayed at 3 days after infection in the tubular structures compared to surrounding non-tubular cells as shown with anti-HSV antibodies. The arrow identifies a rare

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HSV antigen positive tubular cell. These tubular structures are lysed at later days after infection. J. Herpes gene expression is also limited to tumor by in situ hybridization (black grains). K and L. Wild-type virus (17⁺) replicates in brain and tumor and spreads throughout the whole brain. M. H&E of 14 day tumor 18 days after infection with strain 1716. Note the small size of tumor. N. Viral antigen and O. viral gene expression is strikingly limited to the residual tumor mass. Abbreviations: T=tumor; B=host brain

(Scale Bar:=1.2mm in A; =62.5 μ m in B,C,D; =2.0mm in E&G ; =12 μ m in F; = 450 μ m in H, M, N, O; =90 μ m in I; =200 μ m in J: =900 μ m in K and L).

Figure 6: MRI analysis of treated and untreated NT2 tumors.

Nude mice were injected stereotactically with 3×10^4 NT2 cells and 11 days later (d11 post-tumor cell implantation) T1 weighted, gadolinium-enhanced MRIs (A,E) were performed. The presence of a tumor (T) is confirmed by the white enhancing lesion appearing in the superior right hemisphere in these mice. These sections show the area of the maximal tumor mass in cross section. The following day these mice were inoculated with either 5×10^5 PFU of strain 1716 or culture medium. In control mice, the tumor progressed over time and IC volume increased dramatically (B: day 32 post-tumor: C & D: day 41 post-tumor). In strain

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1716 treated mice the tumor regressed and showed no evidence of live tumor cells or virus in the brain (F & G: day 36 post-tumor), (Scale Bar: = 2.77mm in D & G).

Figure 7: Electronmicroscopy

Tumors from nude mice which were mock inoculated or inoculated with strain 1716 were harvested and processed for electronmicroscopy. A: Electronmicrograph of uninfected tumor cells. B: EM of NT2 tumor cells early in infection DNA condensation and viral domains (open arrow) can be observed; C: NT2 tumor cells late in infection, margined chromatin (*) and viral particles (arrowhead) can be observed, D: A dividing cell that is infected, arrow: nuclear membrane, c: cytoplasm, n: nucleus (original magnifications at 2500x).

Figure 8: Prolonged survival of NT2 tumor bearing mice treated with strain 1716.

A: Survival Experiment (Table 1, Study V)- 20 nude mice were stereotactically injected with 3×10^4 NT2 cells. Twelve days later, 10 mice were stereotactically injected with 5×10^5 PFU/ 5μ l of strain 1716 (treated, closed circle) and 10 mice were mock injected with 5μ l of viral culture medium (mock, closed triangle). B: Survival Experiment (Table 1, Study VI)- 17 nude mice were stereotactically injected with 3×10^4 NT2 cells. Ten days later, 9 mice were stereotactically injected 5×10^5 PFU/ 5μ l of strain 1716

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(treated, closed circle) and 8 mice were mock injected with 5 μ l of viral culture medium (mock, closed triangle). C: Weight Graph- Weights of control (closed triangle) and treated mice (closed circles) from Study VI (Table 1; Fig.6B). D: Weights of treated group separated into long-term survivors (HR, closed circle) and dead (LR, closed square) compared with strain 1716 alone treated mice (Study 1, Table 1; closed triangle). Standard Error of the Mean (SEM) bars are included.

Figure 9: Detection of virus in long-term survivors by immunohistochemistry and in situ hybridization.

Long-term survivors were sacrificed and brains and other organs were fixed, sectioned and used for immunohistochemical detection of NT2 cells and HSV and in situ detection of HSV. A: The arrow identifies residual scar at tumor implantation site. B: On histology the brain shows no evidence of any tumor cells (arrow) or replicating virus. C: The residual scar site consists of dystrophic calcifications. This is a higher power view of region identified by the arrow in B. D: Latent virus was observed in the hippocampus (asterisk) of these survivors (4 months post-infection) and the insert shows the nuclear localised signal of LAT positive cells. E: MBP staining shows no evidence of demyelination in the whole brains of these mice. F: Dark-field photomicrograph of in situ hybridization performed

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using a radiolabeled poly(dT) probe to detect total poly (A)*RNA in cells as a measure of metabolic health of the LAT positive cells (asterisk). The experimental tissue (A serial section from 7D) was compared to uninfected, mock infected and RNase treated tissue. There was no detectable difference in the signal in LAT positive area in adjacent serial section. (Scale Bar: =1.2mm in A, B, E; =113 μ m in D and -90 μ m in insert; =113 μ m in F; = 45 μ m in C).

EXAMPLES - SECTION 1Materials and MethodsAnimals:

Female C57B1/6 mice (4 to 6 weeks old - weight approximately 20g) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Tumor Cells:

S91 Cloudman melanoma cells were obtained from the ATCC (Rockville, MD). B16, and Harding-Passey melanoma cells were a generous gift from Dorothee Herlyn (Wistar Institute, Phila, PA). Cells were grown in plastic flasks in AUTO-POW media containing penicillin, streptomycin, and 5% calf serum. When originally obtained, all cell lines were grown up, and then frozen in 95% calf serum/5% DMSO, so that all experiments could be initiated with cells of a similar passage number. On the day of intracranial injection,

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cells in sub-confluent monolayer culture were passaged with 0.25% trypsin solution in EDTA, washed x1 in cell culture media, resuspended at the appropriate concentration in media without serum, and held on ice.

Intracranial tumor production:

Mice were anaesthetized with I.M. ketamine/xylazine (87 mg/kg ketamine/13 mg/kg xylazine). The head was cleansed with 70% EtOH. A small midline incision was made in the skin of the head exposing the skull. Stereotactic injection of tumor cell suspension was performed using a small animal stereotactic apparatus (Kopf Instruments, Tujunga, CA). Injections were done with a Hamilton syringe through a disposable 28g. needle. The needle was positioned at a point 2mm caudal of the bregma and 1mm left of midline. Using a separate 27g. needle with a shield that limits the length of the needle exposed to 0.5mm, the skull was breached at the appropriate coordinates. The injection needle was advanced through the hole in the skull to a depth of 2mm from the skull surface and then backed-out 0.5mm to create a potential space. 1×10^5 cells in a total volume of $2 \mu\text{L}$ were injected over 1 minute. Following the injection, the needle was left in place for 3 minutes, and then slowly withdrawn. The skin was sutured closed.

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Virus:

To produce virus stocks, subconfluent monolayers of baby hamster kidney 21 clone 13 (BHK) cells were infected with HSV strains inl814, 1716, dlspTK, or wild type 17+. Virus was concentrated from the culture and titrated by plaque assay as previously described (28). All viral stocks were stored frozen in viral culture medium (AUTO-POW media containing penicillin, and streptomycin) at -70°C, and thawed rapidly just prior to use.

Viral Inoculation:

Mice were anesthetized with I.M. ketamine/xylazine, and the head was cleansed with 70% EtOH. Using a Hamilton syringe with a 30 gauge disposable needle, the appropriate amount of virus was injected (10^4 - 10^6 PFU in 2 μ L) through a midline incision at the same stereotactic coordinates used for tumor cell injection. The injection was performed over 1 minute, and following the injection the needle was left in place for 3 minutes, and then slowly withdrawn.

Magnetic Resonance Imaging:

Mice were imaged using a 1.9 Tesla 30 cm bore animal MRI system located in the Hospital of the University of Pennsylvania MRI facility. Animals were anesthetized with I.M. ketamine/xylazine (87 mg/kg

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ketamine/13 mg/kg xylazine). Subsequently, each animal was injected with 10 units of Gd(DTPA) via a tail vein. The animal was taped in place within a plexiglass gradient coil and imaged.

Immunohistochemistry:

HSV-infected cells were detected by an indirect avidin-biotin immunoperoxidase method (Vectastain ABC Kit, Vector Labs, Burlingame, CA) as specified by the manufacturer with slight modification. Briefly, tissue sections were deparaffinized, rehydrated, quenched in peroxide (H_2O_2) and blocked in 3.5% goat serum (Sigma Chem. Co., St. Louis, MO.). Tissue sections were incubated overnight at 4°C with the primary antibody, a rabbit antiserum to HSV-1 (Dako Corp., Carpinteria, CA), used at a dilution of 1:1000. Next, the tissue was incubated at room temperature with biotinylated goat anti-rabbit IgG, the avidin-biotin horseradish peroxidase complex and finally AEC substrate. Sections were counter stained with hematoxylin and examined under the light microscope. As a control for the specificity of immuno-staining, tissues were processed as above, except that non-immune rabbit serum was substituted for the primary HSV-1 antiserum.

Titration of virus from tumor and brain:

Mice were sacrificed by lethal injection of

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anesthesia. Brains with or without in situ tumors were removed aseptically, snap frozen in liquid nitrogen, and stored at -70°C. Each tissue sample was rapidly thawed in a 37°C water bath, and the tissue was homogenized in viral culture medium at a 10% weight/volume ratio using a Pyrex Ten Broeck tissue grinder. The homogenates were centrifuged at 3,000 x g for 10 minutes at 4°C. The supernatant of each tissue homogenate was diluted logarithmically in media, and the viral titer of each was determined by plaque assay on BHK cells (28).

Statistics:

Standard deviation, and t-Test: two sample assuming unequal variances, were calculated using Microsoft Excel (Redmond, WA) on an apple MacIntosh computer (Cupertino, CA).

EXAMPLE 1 (lysis of melanoma cells)

In our initial studies, we wanted to make a straightforward in vitro determination of the relative abilities of HSV-1 wild type and mutant viruses to lyse various murine melanoma cells. We also wanted to compare how efficiently these melanoma cells were lysed by HSV-1 relative to baby hamster kidney (BHK) cells, which is a standard cell line used to propagate and titer HSV-1. Cells were plated in 24 well tissue culture plates at a density of 5×10^4 cells/well. The

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viruses were diluted logarithmically and cell monolayers were infected in triplicate. After 72 hours of culture, the highest dilution of virus at which complete destruction of the monolayer still occurred, was recorded for each virus-cell combination. Data are expressed as the number of PFU of virus, obtained for each virus-cell combination.

As demonstrated in Table 1, the various mutant viruses lyse melanoma cells and BHK with efficiencies similar to wild type 17+. Cloudman S-91, and H-P melanoma cells were lysed efficiently relative to BHK.

EXAMPLE 2 (tumor production)

The capacity of each melanoma cell line to produce intracranial tumors was then evaluated. For each cell line, 10 C57B1/6 mice were injected stereotactically with 5×10^4 cells in the right cerebral hemisphere. Mice were observed daily, and sacrificed when they appeared moribund, or after 6 weeks if they remained asymptomatic. Each brain was fixed, sectioned, stained, and examined histologically for tumor. Both H-P and B-16 formed intracranial tumors in 10 of 10 C57B1/6 mice, while Cloudman S-91 only formed a tumor in 1 of 10 mice.

We decided to proceed with the H-P model, since these cells were both susceptible to lysis by the relevant HSV-1 mutants, and formed brain tumors efficiently.

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Subsequent experiments verified that stereotactic injection of H-P cells into the brain of C57B1/6 mice establishes tumors in 100% of the animals. A technical advantage of this system is that the presence of a brain tumor can be verified by magnetic resonance imaging (MRI) prior to treatment, or simply by observation of a pigmented area on the skull overlying the tumor site, generally by 5 days post cell injection. The tumors progressed to a size that caused the mice to become moribund from neurologic symptoms in approximately two weeks.

EXAMPLE 3 (treatment of brain tumors with HSV-1 mutant 1716).

C57B1/6 mice were injected stereotactically in the right cerebral hemisphere with 5×10^4 Harding-Passey melanoma cells. After 10 days (Figure 1a) or 5 days (Figure 1b), 5×10^5 PFU of HSV 1716 was injected at the same stereotactic coordinates. The number of days elapsed between injection of tumor cells and time mice became moribund is shown on the X axis. Control mice were injected with an equal volume of viral culture medium at the appropriate time.

As shown in Figure 1a stereotactic injection of HSV-1 mutant 1716 into brain tumors 10 days after establishment, resulted in a statistically significant increase in the length of time elapsed until the mice become moribund ($P(T \leq t)$ one-tail: 1.016×10^{-4}).

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However, no long term survivors were obtained. When viral therapy was performed 5 days after tumor establishment (Figure 1b), significant improvement in outcome was again seen in the treatment group ($P(T \leq t)$ one-tail: 7.707×10^{-3}), and 2/10 treated mice were cured. One long term survivor was sacrificed after day 39 post viral infection. Microscopic examination of serial sections of the brain did not reveal any residual tumor (data not shown). The second animal is still alive and asymptomatic at greater than 150 days post treatment. Treated animals that became moribund, showed progression of their brain tumors upon examination of tissue sections.

EXAMPLE 4 (1716 replication in tumor and non-tumor cells).

Immunohistochemistry shows that replication of 1716 is in fact restricted to tumor cells, and does not occur in surrounding brain. A significant number of melanoma cells within tumor were stained by polyclonal antiserum to HSV-1 on days 3 and 6 post infection. Moreover, in tumor bearing mice treated with 1716, no HSV-1 antigen staining was seen in brain tissue adjacent to tumor or in any other areas of brain in all sections examined. In addition, no histologic evidence of encephalitis was seen in any 1716 treated mice at any time. In contrast, tumor bearing mice infected with wild type 17+ virus,

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exhibited multiple focal areas of HSV-1 immunohistochemical staining both within tumor and in surrounding and distant brain as well. A significant encephalitis characterised by polymorphonuclear leukocytes, nuclear dust, and extravasation of red blood cells, is seen in areas of this and other sections examined. In control experiments, no immunohistochemical staining was seen with anti-HSV-1 in tumor or brain from mice who did not receive virus, or in virally infected brain tumor sections subjected to the full immunohistochemical protocol with normal rabbit serum substituted for the primary anti-HSV-1 antibody (data not shown).

EXAMPLE 5 (kinetics of replication in tumor and non-tumor cells).

Having shown striking restriction of 1716 replication to tumor by immunohistochemistry, we next attempted to quantify the kinetics and extent of replication of 1716 in tumor by titration of infectious virus, and compare this with titration data from non-tumor bearing mouse brain infected with 1716 or 17+.

To investigate the extent of 1716 replication in brain tumors, C57B1/6 mice were injected with Harding Passey melanoma cells right of midline. Seven days later each mouse was infected with 5×10^5 PFU of 1716 at the same stereotactic coordinates. At the times

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indicated, mice were sacrificed, the brains were frozen in liquid N₂ and stored at -70°C. Specimens were thawed rapidly, homogenized, and viral titration was performed in triplicate on BHK cells (closed squares). These data represent the mean of 4 mice at each time point.

To establish the growth characteristic of 1716 and wild type 17+ in brain without tumor, mice were injected intracranially with either 5×10^5 PFU 1716 (open squares) or 1×10^3 PFU of 17+ (closed triangles). Mice were sacrificed at the times shown and processed as described above. Each point is the mean of 2 mice.

As shown in Figure 2, wild type 17+ virus replicated efficiently in non-tumor bearing mouse brain. In contrast, no replication of 1716 occurred in brain of non-tumor bearing mice. The titer of virus recovered decayed over time, and infectious 1716 could only be isolated for 3 days after inoculation. However, when 1716 was injected into brain tumors, significant replication occurred as evidenced by recovery of an amount of infectious 1716 on day 1 post inoculation that is substantially greater than the input amount. Under these conditions, infectious 1716 could be isolated from tumor bearing mice for 5 days post inoculation, but not on day 7. These results clearly demonstrate that HSV-1 mutant 1716 will freely replicate in tumor cells (leading to their

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destruction) but does not replicate in non-tumor cells (leaving them unharmed).

Table 1: Relative susceptibility of melanoma cells to lysis by HSV-1.

<u>Virus</u>	Cloudman S91	<u>Cell Type</u>	
		Harding- Passey	BHK
<u>dlspTK</u>	10^3	10^4	$> 10^3$
1716	10^4	10^4	$- 10^3$
17+ (wild type)	10^3	10^3	$> 10^2$

EXAMPLES - SECTION 2

MATERIALS & METHODS

Virus Stocks

To produce virus stocks, subconfluent monolayers of baby hamster kidney 21 clone 13 (BHK) cells were infected with HSV strains 1716, in1814, or parental 17+. Strain in1814 has a mutation (insertion) in the VP16 gene (located in the U_L region; Figure 3A) and strain 1716 has a mutation (deletion) in the $\gamma 34.5$ gene (mutant; Figure 3C). Virus was concentrated from the culture, titered on BHK cells by plaque assay and stored at -70°C in 0.5ml aliquots of viral culture medium (AUTO-POW media containing penicillin and streptomycin) and thawed rapidly just prior to use as described (30,34).

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25Culture of Tumor Cells and Differentiation of NT2

NTera-2 (clone D1) cells (referred to here as NT2 cells) were cultured as described (28,29). Briefly, the cells were passaged 1:3 twice per week in OptiMEM with 5% fetal bovine serum (FBS) and penicillin/streptomycin (P/S). The medulloblastoma cell lines, D283 MED and DAOY, were cultured in RPMI 1640 with 10% FBS, 1% P/S and 1% Glutamine. BHK cells were cultured in AUTO-POW with 5% FBS, 1% P/S and 1% Glutamine. NT2 cells were plated at a density of 2.0×10^6 in T75 flask, and fed twice weekly with DMEM-HG supplemented with 10% FBS, 1% P/S, and 10^{-5} M retinoic acid for 5 weeks. The differentiated NT2N cells were separated from non-neuronal cells as described (29,35). On the day of intracranial injection, NT2 cells in sub-confluent monolayer culture were harvested, washed three times in buffer and placed on a bed of ice until injected into the brains of nude mice.

Plaque Assay

NT2, BHK, DAOY and D283 MED cells were plated in 24 well tissue culture plates at a density of 10^5 cells/well. The viruses of interest were diluted logarithmically and cell monolayers were infected in triplicate with multiplicity of infections (MOI) ranging from 10 to 0.01. Cultures were observed regularly for the degree of cytopathic effects (CPE)

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of the viruses and noted for each MOI.

Titration of Virus from Cell Cultures

Cells were infected at MOI=1, harvested at 4, 24 and 48 hrs post-infection and stored at -70°C. The samples were freeze-thawed twice from -70°C to 37°C, centrifuged at 3,000 x g for 10 minutes at 40°C, the supernatant was diluted logarithmically in media and the viral titer of each sample was determined by plaque assay on BHK cells (34).

Titration of Virus from Tumor and Brain

To titrate viral inoculums in tumor and brain, nude mice were intracranially inoculated with 1×10^6 PFU of strain 17* or 6.25×10^4 PFU of strain 1716 and the mice were sacrificed by lethal injection of anesthesia (ketamine/xylazine). The brains and tumors were dissected from mice that were sacrificed on different days post viral inoculation (day 0, 1, 3, 5 and 7), quick frozen in liquid nitrogen and stored at -70°C. The brain and tumor samples from the different time points were rapidly thawed in a 37°C water bath, and the tissue was homogenized in viral culture medium at a 10% weight/volume ratio using a Pyrex Ten Broeck tissue grinder. The homogenates were centrifuged at 3,000xg for 10 minutes at 4°C, the supernatant was diluted logarithmically in media and the viral titer of each sample was determined by plaque assay on BHK

cells (34).

Intracerebral Graft Implantation

Female homozygous nude mice (4 to 6 weeks old) were obtained from Harlan Sprague Dawley (Indianapolis, IN), the mice were anesthetized with intramuscular (IM) ketamine/xylazine (87 mg/kg ketamine/13mg/kg xylazine) and stereotactic injections of tumor cell suspensions were performed using a small animal stereotactic apparatus (Kopf instruments, Tujunga, CA), a 10 μ l Hamilton syringe and a 30 gauge disposable needle as previously described (35). To make cortical tumors, the syringe needle was positioned at a point 2mm rostral of the bregma and 1mm to the right of midline. The skull was cleansed with 70% ethanol and perforated with a 27 gauge needle and the Hamilton syringe with the attached needle was advanced through the hole in the skull to a depth of 1.5mm below the dura and 3 x 10⁴ NT2 cells in a total volume of 2 μ l were injected over 5 min. Prior to implantation, NT2N cells were suspended in DMEM/HG and maintained at 40°C in an ice bath. Exactly 5 μ l of the NT2N cell suspension, containing approximately 5 x 10⁵ cells, was injected at the same location as above. Following the injection, the needle was left in place for 5 min and then slowly withdrawn over 2 min. and the superficial skin wound was closed with sutures. The mice were allowed to recover and inspected daily

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for signs of illness. Body weight and cranial measurements with calipers were taken weekly. Any mice that showed signs of morbidity in extremis were sacrificed and brains were prepared for histochemistry. Tissues from some animals that died unobserved in their cage also were harvested and fixed for histochemical analysis. The experiments on nude mice are summarised in Table 2.

Viral Inoculation

Control mice and mice previously inoculated with tumor cells were anesthetized as described above and the head was cleansed with 70% ethanol. Using a Hamilton syringe with a 30 gauge disposable needle, the appropriate amount of virus was injected (10^4 - 10^6 PFU in 5 μ l) through a midline incision at the same stereotactic coordinates used for the previous injection of tumor cells. The injection was performed over 3 min following the injection and then slowly withdrawn over 1 min. Control mice received equivalent volume inoculations of viral medium.

Magnetic Resonance Imaging

The brains of selected mice were imaged using a 30 cm bore 1.9 Tesla animal Magnetic Resonance Imaging (MR1) system (General Electric). To accomplish this, mice were anesthetized as described above at various times after implantation of tumor cells and

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inoculation of the tumor sites with virus. Subsequently, each animal was injected with 10 units of an enhancing agent, gadolinium complexed to a DTPA carrier (Magnevist), via a tail vein. The animal was then immobilised within a Plexiglas RF coil and imaged.

Immunohistochemical Procedures

Mice were transcardially perfused and fixed with 70% ethanol in isotonic saline (150mM NaCl, pH 7.4) or 4% paraformaldehyde (0.1M PBS, pH 7.4) and the brain as well as samples of multiple other tissues (i.e. trigeminal ganglions, heart, proximal jejunum, liver, spleen, left kidney, femur, and vertebral bodies) were dissected for histological and immunohistochemical analysis. The methods for tissue processing and light microscopic immunohistochemical analysis were similar to those described elsewhere (35,36). Both monoclonal and polyclonal antibodies to neuronal and glial cytoskeletal proteins and other polypeptides that have been shown to serve as molecular signatures of the neuronal or glial phenotype were used for the immunohistochemical characterisation of intracranial allografts (35,37). Rabbit polyclonal antisera to HSV-1 which detects the major glycoproteins present in the viral envelope and at least one core protein (Dako Corp., Carpinteria, CA) was used at a dilution of 1:1000 to detect replicating virus (38). A mouse

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monoclonal antibody (MOC-1) to neural cell adhesion molecule (NCAMs) specific for human NCAMs was used at a dilution of 1:100 to detect NT2 and NT2N cells and to distinguish them from mouse brain cells (35). Another monoclonal antibody, RMO93 (1:10), which recognises rodent specific epitopes of the middle molecular weight neurofilament (NF-M) protein and does not cross-react with human NF-M was used to confirm the identity of NT2N grafts (35). RMO301 (1:100) is a monoclonal antibody that recognises human specific NF-M was used to confirm NT2N grafts. M13, a mouse monoclonal antibody which recognises human microtubule associated protein-2 (MAP2), was used at a 1:500 dilution. Rabbit polyclonal antibody specific to mouse myelin basic protein (MBP) was used at a dilution of 1:1000 (gift of A. McMorris). Tissue sections for staining with M1B-1 (a mouse monoclonal antibody that recognises a cell-cycle specific antigen (Ki-67) used at a 1:20 dilution; AMAC, Westbrook, ME) were pretreated by microwaving on 10mM Sodium Citrate as described (39). Prior to sacrifice some control mice were injected with intraperitoneally with bromodeoxyuridine (BrdU) at 5mg/g (in 150mM NaCl, 7mM NaOH) body weight in order to label NT2 cells undergoing cell division in the grafts as described (37). Segments of the proximal intestine were removed from the same mouse as positive controls for cycling cells. BrdU positive cells were identified by using

an BrdU antibody BU-33 (1:250). Antigen expressing cells were detected by the indirect avidin-biotin immunoperoxidase (Vectastain ABC kit, Vector Labs, Burlingame, CA) or peroxidase anti-peroxidase detection systems with 3,3'-diaminobenzadine (DAB) as the chromagen. Grafts and spread of virus in all animals was monitored by screening every tenth section through entire brain with MOC-1, MIB-1 and HSV antibodies.

In Situ Hybridization for HSV-1 Specific Gene Expression.

Sections of perfused and fixed tissue were mounted on slides and in situ hybridization was performed as previously described to detect viral gene expression (26,33,40). Serial tissue sections were hybridized with a ³⁵S-labeled HSV LAT specific probe (BstEII-BstEII, Fig.1E), with a ³⁵S-labeled HSV specific thymidine kinase probe (tk; an early gene product) or with a biotinylated HSV specific gC (a late gene product) probe.

Preparation of ³⁵S-labeled Nick-Translated Probe

The latency-associated transcript (LAT) probe BstEII-BstEII subfragment (0.9kb) of BamHI B was isolated from restriction digests by gel electrophoresis and purified by GeneClean (Bio 101 Inc.; La Jolla, CA; see Fig.3) (30). The 3.4kb BamHI fragment encoding the tk gene was isolated as

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described (41). DNA probes were nick-translated and separated from unincorporated nucleotides by passage through Sephadex G-50 spin columns (Pharmacia) (33). The specific activities of the probes were approximately $1-5 \times 10^8$ c.p.m./ μ g DNA.

Preparation of 35 S-labeled Poly (dT) Probe

A 21-mer of poly(dT) was synthesized and was used as substrate for labeling by terminal deoxynucleotidyl transferase (TdT). Reaction mix consisted of 2 μ l of TdT, 1 μ l of Poly(dT) (6 μ g/ μ l), 5 μ l of 5X TdT Buffer, 6 μ l of CoCl_2 (2.5 mM), 10 μ l of ^{35}S -dTTP (1 μ g), 1 μ l of ddH_2O . The mix was incubated at 37°C for 30 min. and was separated from unincorporated nucleotides by passage through Sephadex G-25 spin columns. In situ hybridization was performed exactly as above except that hybridization and washes were performed at 37° in 25% formamide (42). Exposure time courses were performed on uninfected, mock infected, wild-type virus infected and RNase treated tissue sections and were used as positive and negative controls for experimental tissue sections (see 43).

Biotinylated gC Probe to Detect Active Viral Replication.

A nonisotopic in situ hybridization was performed using a 21 bp antisense gC probe (nucleic acids 199-219 of gC transcript, CGGGGCGGGGGTGGCCGGGGG; gift by

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K. Montone; Fig.3F) linked to the 3' end by a biotinylated tail [5'-(TAG)₂-BBB-3']. The protocol was essentially the same as in Wang and Montone with slight modifications for mouse brain tissue.

DNA Nick End Labeling By TUNEL Method

The terminal deoxynucleotidyl transferase (TdT) dUTP-biotin nick end labeling (TUNEL) technique was performed as previously described (37). Briefly, deparaffinized and rehydrated slides were digested with 20µg/ml of proteinase K in 0.1 M Tris (pH=8) at room temperature for 15 min. After washing, the sections were incubated with a mixture containing 20mM biotinylated-dUTP, 0.3 U/µl terminal deoxynucleotidyl transferase, 1.5mM cobalt chloride, 200mM sodium cacodylate, 25mM Tris, 0.25 mg/ml bovine serum albumin (pH=6.6) at 37°C for 45 min. The reactions were stopped by washing in 2X SSC for 15 min. and the results were visualized by alkaline phosphatase conjugated with streptavidin and developed with Fast Red substrate. Coronal sections of post-natal day eight rat brain were used as positive controls for this TUNEL protocol because this was the developmental stage at which peak apoptosis activity was recognised (44).

Electron Microscopy of Thin Sections.

Portions of tumor tissues from perfused mouse

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brains were fixed in 1% glutaraldehyde, 4% paraformaldehyde in 0.1M sodium cacodylate (pH=7.4) over night at 4°C and washed in sodium cacodylate buffer and processed for EM as described (37).

Statistics

Survival and weight statistics were performed using BMDP Statistics Software (ed. WJ Dixon; Release 7.0; 1993). Differences in survival in control and treated groups were compared using Generalised Wilcoxon (Breslow) Analysis. Differences in weights were compared using the t-test and the Mann-Whitney Test. Moribund animals in extremis were sacrificed and treated the same as animals found dead in their cage for statistical analysis.

EXAMPLE 6:HSV-1 Strain 1716 Lyses and Spreads on Monolayers of Tumor Derived Human Neural Cell Lines In Vitro.

To determine how efficiently HSV-1 strain 1716 lyses rapidly dividing NT2 in comparison with parental strain 17⁺, NT2 cells were plated on 24 well plates 1 day prior to infection by these two strains at Multiplicities of Infection (MOI) of 10, 1 and to 0.1. Both viruses, at MOI of 10, lysed NT2 cells within 1 day and this was associated with the characteristic morphological changes (rounding up, phase brightness, cytomegaly, plaque formation and loss of adhesion)

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associated with HSV infection. Since the behaviour of a virus at a low MOI (0.1) in vitro may predict the ability of a virus to spread in a tumor in vivo, we studied infection at MOI-0.1. Strain 1716 spread and destroyed monolayers of NT2 cells less efficiently than 17⁺ (1716 lysed monolayer in 3 days and 17⁺ in 2 days). The behaviour of these viruses was similar in two different human medulloblastoma cell lines (D283 MED and DAOY) suggesting that strain 1716 can lyse many different brain tumor cell lines.

Next, NT2N cells (the neuron-like retinoic acid differentiated derivatives of NT2 cells) were infected with these viruses. Strain 1716 was attenuated for cytopathicity in these cells with respect to strain 17⁺ and Lactate Dehydrogenase (LDH) assays for cytotoxicity performed on NT2N cells infected with the above viruses showed that both viruses caused some non-specific toxicity within 12 hours after infection (data not shown). Interestingly, titration of virus from infected cell cultures showed that strain 1716 was deficient for replication in NT2N cells (data not shown). Because strain 1716 is more severely neuroattenuated in mice than the other engineered strains (26, 30), we conducted in vivo studies of strain 1716 versus 17⁺ virus inoculated into the CNS of nude mice with and without transplants of in vitro derived NT2N cells or with tumors established from transplanted NT2 cells.

Reference is made to Table 2.

EXAMPLE 7:

Replication of HSV-1 Strain 1716 Cannot be Detected In
The Mouse CNS Following Intracerebral Inoculation

Consistent with previous results using SCID mice (26), intracerebral (IC) inoculation of 5×10^6 plaque forming units (PFU) of strain 1716 in nude mice did not induce clinical symptoms for over 4 months post-inoculation, and there was no evidence of encephalitis on histological analysis of the brains nor any evidence of replication in the major organs (e.g. liver, spleen, bone marrow, etc.) of these mice (Table 2, Study I). In contrast to strain 1716, IC inoculation of less than 100 PFU of strain 17⁺ killed nude mice within 5-10 days and histopathological analysis revealed extensive cytopathic lesions (e.g. intranuclear inclusion bodies, cell death, etc.) in these mice (not shown).

To monitor viral replication in the brain after IC inoculation of strain 1716 versus strain 17⁺, a viral titration assay was performed (Table 2, Study II). The Recovery of both viruses on day 0 was low relative to the amount of virus in the injected inoculum. This probably was due to adsorption or fusion of the viral particles to the membranes in the brain homogenates and to inactivation of virus during harvesting. Figure 4 shows that the titer of strain

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17⁺ exponentially increased with time and resulted in morbidity and death of inoculated mice. In contrast, the titer of strain 1716 dropped precipitously in the brains of nude mice, and virus was no longer detectable 3 days post-inoculation. Moreover, there was no immunohistochemical evidence of encephalitis in strain 1716 infected mice, and there was no detectable spread of strain 1716 virus outside the CNS as evidenced by the absence of virus in samples of liver, spleen, kidney, jejunum and bone marrow by immunohistochemistry and by in situ hybridization for HSV specific transcripts (data not shown). Likewise, direct inoculation of liver or intravenous injection with strain 1716 did not cause any morbidity or death in nude mice. In contrast to strain 1716, strain 17⁺ infected mice exhibited evidence of encephalitis and tumor lysis (see Figure 4).

EXAMPLE 8:HSV-1 Strain 1716 Lytically Replicates In NT2 Tumors
But Not in Transplanted NT2N cells In The Mouse CNS

When strain 1716 was injected into NT2 tumors significant replication occurred for 7 days as evidenced by increase in viral titer over input inoculum by day 3 (Fig.4). This is in agreement with the immunohistochemical and in situ hybridization data which showed no detectable strain 1716 in the brains of these mice except in the NT2 tumor cells for as

long as they were present to support viral replication (see below).

Since quantification by titration assay in mice showed that strain 1716 replicated in NT2 cell tumors, we tested the ability of strain 1716 to induce regression of these tumors (Table 2, Study IV). To do this we injected 5 μ l of strain 1716 containing 5×10^5 PFU into tumors that formed in the brains of nude mice following IC implantation of 3×10^6 NT2 cells. To monitor the fate of transplanted NT2 cells, mice were sacrificed at different time points after the viral inoculation and their brains and organs were analysed by immunohistochemistry and in situ hybridization. NT2 cells formed tumors with a neural and epithelial histology in 100% of mice, and these tumors were lethal within 5 weeks after grafting (Fig.5A, 5B). These tumors contained abundant proliferating cells as evidenced by BrdU labeling, the immunohistochemical detection of cell cycle antigens and their rapid growth (Fig.5C, 5D). Figures 5E,F,G and H show that infection of NT2 tumors with strain 1716 is not uniform at day 3 post-infection (pi). This may reflect the localised nature of the injection site, or to cell-type specific differences in the vulnerability of the cells to infection and lysis by strain 1716. Nonetheless, most tumor cell types near the injection site harbored immunoreactive virus by day 5. At later days post-infection, more cells were infected, but

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immunoreactivity for virus in the tumor was weaker presumably due to clearance of the virus following lysis of the infected tumor cells (Figure 5).

As seen in Figure 5H, viral antigen is limited to the tumors. On high magnification, the infected tumor cells showed characteristic features of HSV-1 infection, i.e. intranuclear inclusion bodies and multinuclear giant cell formation (Fig.5F). No viral antigen staining was seen in the surrounding brains of these mice or in the brains of control untreated mice. This was confirmed by a non-isotopic in situ hybridization protocol using a biotinylated probe for glycoprotein C (gC) and a radiolabeled thymidine kinase (TK) probe (a late and early gene product expressed only in acute infection) to detect active viral replication in serial sections (Fig.5J). Thus, viral replication, as evidenced by gene expression, is also restricted to the tumor cells. In contrast, tumor-bearing mice inoculated with strain 17⁺ showed viral replication in both tumor and brain (Fig.5K, 5L). Mouse brains harvested 18 days after viral treatment of their tumor implants with 1716 showed a marked decrease in the size of tumor. Indeed only a residual fibrotic scar was seen in some mice, and viral antigen was strictly limited to the remaining cells in the scar (Fig.5 M, N, O).

To examine the ability of strain 1716 to induce regression of brain tumors, NT2 tumors in the brains

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of nude mice were stereotactically injected with strain 1716 twelve days after implantation of NT2 cells. Treated mice were inoculated with 5×10^5 PFU (in $5\mu\text{l}$) of strain 1716 at the tumor implantation site and control mice received $5\mu\text{l}$ medium alone. MRI scans showed that these mice developed detectable tumors by 11 days post-implantation. In all mock treated tumor-bearing mice, the tumor grew rapidly in size and was lethal (Fig.6A-D). In all treated mice, strain 1716 infection induced a detectable regression of the tumor at the original inoculation site (Fig.6E-G).

To determine whether transplanted NT2N cells were permissive for HSV replication, 2.5×10^5 cells were transplanted into the brain parenchyma or ventricles of nude mice (Table 1; Study III). These cells integrate and survive for over a year and acquire a fully mature post-natal human CNS neuronal phenotype (27). Strain 1716 was then inoculated at the same stereotactic site 6 weeks post-implantation. The grafts were identified and distinguished from mouse cells by using human specific (MOC1 and RM0301) and mouse specific (RM093) antibodies to neural cell adhesion molecule (MOC1) or neurofilament proteins (RM0301, RM093). In contrast to NT2 tumors, these long-term NT2N transplants were non-permissive for strain 1716 replication as evidenced by lack of immunohistochemical staining for viral antigens at days 1,3,5,7,9,21 and 50 post-viral inoculation.

EXAMPLE 9:HSV Strain 1716 Induces A Non-Apoptotic Death In NT2 Tumor Cells.

Sections of tumor from a selected group of strain 1716 infected mice and uninfected tumor controls were prepared for EM analysis to characterise the mode of cell death in the NT2 cell tumors. The infected cells on H&E staining had the characteristics typical of HSV infected cells (Fig.5F). Viral assembly domains can be seen in the nucleus of infected cells. There was no evidence for an apoptotic mechanism of cell death in the virally infected tumor cells by EM (Fig.7). Tumor cells with viral particles showed the fragmentation and dissolution of nuclei and organelles as well as condensed and margined DNA. Terminal deoxynucleotidyl transferase (TdT)dUTP-biotin nick end labeling (TUNEL) and DNA gel electrophoresis studies did not show evidence of DNA fragmentation indicative of apoptosis (data not shown). Taken together, these findings indicate that strain 1716 induces the characteristic lytic infection in the NT2 cells in vitro and in vivo.

EXAMPLE 10:Long-term Survival of HSV Strain 1716 Treated Tumor-Bearing Mice.

Based on the results of the studies described above, we analysed the survival of a cohort of tumor-bearing

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mice that were or were not treated with virus (Table 2, Studies V and VI). This also enabled us to assess the long-term consequences of treatment with strain 1716 (see below). Twenty mice were inoculated with 3×10^6 NT2 cells and several days later mice were split into two groups. Control mice received culture medium and treated mice received 5×10^6 PFU of strain 1716. In the first survival experiment (Table 2, Study V) virus treatment was given at 12 days post-tumor cell inoculation. There was only 1 (10%) long-term survivor in this group and there was no significant difference in survival between control (Study VA) and treated (Study VB) animals ($p=.63$, Fig.8A). Histological examination of some control and treated animals showed that virus was replicating in the tumor. In the treated animals it appears that the tumor had already grown and spread to such a size that one virus treatment was not sufficient to induce regression of the tumor (data not shown). When virus treatment was given at 7 days post-tumor cell inoculation, 44% of the mice survived long-term and 100% of control mice died within 10 weeks (Table 2, Study VI; Fig.6B). This resulted in a statistically significant improvement in survival ($p<.03$; median survival time (controls)= 44.75 ± 5.24 ; median survival time (treated)= 101.78 ± 22.69).

In the survival experiments, significant weight loss was observed in a sub-population of both mock-

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treated tumor-bearing mice (Table 2, Study VIA) and strain 1716 treated tumor-bearing mice (Table 2, Study VIB). Since there was no difference in the average weights of these two groups (Fig.8C) we separated the treated group (Study VIB) into two sub-groups (High-Responders (HR)-the long-term survivors (4 mice) and Low-Responders (LR)- mice that died (5 mice); Fig 8C). We found a significant difference in weight loss at 6 weeks ($p < 0.01$) between groups HR and LR and between groups LR and 1716 treated mice (Study 1) using the standard t-test (Fig.8D). These data confirmed earlier observations of weight loss in Study V, and they suggest that the weight loss may be due to the toxic physiological effects of tumor growth, regression or lysis and not directly due to an effect of the viral infection of the brain. Notably, some of the treated mice that died showed leakage of tumor cells from implantation site into ventricles and leptomeninges. Since this would lead to obstruction of the flow of cerebrospinal fluid, it is not surprising that some of these treated mice had survival kinetics similar to control mice (Fig.8A). Finally, intracranial volume of the control mice with brain tumors increased by over 25% (indicating tumor growth) while in treated mice intracranial volume did not increase significantly (see Fig.6). The long-term survivors also did not have a significant increase in intracranial volume (data not shown). These

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interpretations are supported by the fact that mice inoculated with strain 1716 alone did not show any clinical or histological characteristics of encephalitis at any time during the study

Long-term survivors from studies V and VI (Table 2) had no clinical symptoms, no atypical increase in intracranial volume, and no weight loss (Fig.8C). These mice and mice inoculated with strain 1716 alone were sacrificed and analysed for pathology and viral replication by immunohistochemistry and in situ hybridisation. In the survivors, there was only evidence of fibrotic scar tissue and dystrophic calcifications but no evidence of residual, live tumor cells (Fig.9A,B,C). Immunohistochemical staining for cell cycle antigens (e.g. using MIB-1) was also negative suggesting the absence of any cycling NT2 cells in the brain. Further, the brains of these mice were negative for herpes antigens indicating the absence of replicating virus, although in some mice in situ hybridization revealed the presence of latent HSV in the hippocampus (Fig.9D). Surprisingly, latent virus was also found in the hippocampus of mice infected with strain 1716 along. However, examination of representative rostral to caudal levels of the brains of all survivors using antibodies specific to HSV and to the human NT2 cells (MOC-1, RMO301), did not reveal any evidence of active viral replication nor any residual live tumor cells. To exclude the

possible occurrence of other potential toxic sequelae of HSV-1 strains such as demyelination, we probed sections from the brains of mice using antibodies to myelin basic protein. Examination of mice injected with strain 1716 alone as well as long-term survivors revealed no evidence of demyelination (Fig.9E). Finally, we monitored the levels of poly(A)⁺RNA by in situ hybridisation using a radiolabeled poly(dT) probe to assess the overall metabolic health of neurons (31,32), and we found no quantifiable difference in the level of poly (A)⁺RNA between the LAT-positive cells of the long-term survivors versus the contralateral non-LAT positive cells in the same mice and in uninfected, control mice (Fig.9F).

TABLE 2: Summary of Animal Experiments

Study	Treatment		Survival	Clinical Disease
	Tumor	Virus (p.i.) ^a Number		
I.	-	1716 4	100% (>16wks)	n.a.
II.A	-	17 ⁺ 10	n.a.	Encephalitis
B	-	1716 10	n.a.	n.a.
C	NT2	1716(12) 10	n.a.	n.a.
III.	NT2N	1716(6wks) 10	n.a.	None
IV.A	NT2	- 10	b	None
B	NT2	1716(14) 10	b	None
C	NT2	17 ⁺ (14) 2	b	Encephalitis
V.A	NT2	- 10	0%(<8wks)	Cachexia ^c
B	NT2	1716(12) 10	10%(>35wks) ^c	None ^d
VI.A	NT2	- 8	0%(<10wks)	Cachexia ^c
B	NT2	1716(7) 9	44%(>25wks) ^f	None ^d

^ap.i.-post cell implantation when virus or mock inoculum was administered. n.a. - not applicable.

^bmice were sacrificed at different days after viral inoculation to follow the kinetics of tumor progression and viral spread (see text for details).

^cThese mice also showed no symptoms but the mice that died showed the same range of symptoms as in mock treated mice.

^dLong-term survivors showed no symptoms but the mice that died showed same range of symptoms as in mock treated mice.

^ep=0.63, no statistically significant difference in survival between mock and treated mice.

^fp<0.03, statistically significant difference in survival between mock and treated mice.

CONCLUSION

HSV type I (HSV-1) strain 1716 has a deletion in the γ 34.5 neurovirulence gene which renders it avirulent in the mouse CNS, we have assessed its potential to induce selective lysis of tumor cells versus neurons in vitro and in vivo. Parental HSV-1 strain 17⁺ and engineered strain 1716 were studied using human teratocarcinoma derived embryonal carcinoma cells (NT2 cells). These cells resemble neuronal progenitor cells and can be induced to differentiate into neurons (NT2N cells) with retinoic acid. Intracerebral grafts of NT2 cells into the brains of nude mice resulted in lethal brain tumors while grafts of NT2N cells resulted in the integration and maturation of NT2N cells without neoplastic reversion. In vitro studies showed that strain 1716 replicates in and spreads on monolayers of NT2 cells, resulting in the lysis of these cells. However, strain 1716 did not replicate in NT2N cells in vitro. In vivo, strain 1716 replicated preferentially in NT2 tumors as evidenced by immunohistochemical staining for viral antigens, in situ hybridisation for HSV specific transcripts and by titration of virus from brains with tumor following intracranial injection of the virus into these mice. In contrast to NT2 tumor cells, transplanted NT2N cells were non-permissive for strain 1716 replication. The temporal regression of NT2 tumors in mice treated with strain 1716 was

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demonstrated in vivo by Magnetic Resonance Imaging. Electron microscopy and studies of DNA fragmentation suggested that regression of NT2 brain tumors in strain 1716 treated mice was mainly due to a non-apoptotic, lytic mode of cell death. Strain 1716 treated NT2 tumor-bearing mice survived over twice as long as mock-treated tumor bearing mice and these differences in survival (25 vs.9 wks.) were statistically significant ($p < .03$). We conclude from these studies that strain 1716, a replication-competent, non-neurovirulent mutant of HSV-1, induces regression of human neural tumors established in the brains of nude mice resulting in their prolonged survival. These results indicate that HSV-1 $\gamma 34.5$ mutants are candidates for the treatment of human brain tumors in vivo.

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CLAIMS

1. Use as an anti-cancer agent of a mutant herpes simplex virus type HSV-1 wherein the mutant virus is a mutant strain 17 virus and comprises a modification in the γ 34.5 gene in the long repeat region (R_L) such that the γ 34.5 gene is non-functional.
2. Use of a mutant herpes simplex virus according to claim 1 wherein the virus is substantially non-neurovirulent.
3. Use of a mutant herpes simplex virus according to any preceding claim wherein the modification to the virus is made within the Bam H1 s restriction fragment of the R_L terminal repeat.
4. Use of a mutant herpes simplex virus according to claim 3 wherein the modification is a deletion of from 0.1 to 3kb, in particular of from 0.7 to 2.5 kb.
5. Use of a mutant herpes simplex virus according to claim 4 wherein the deletion is a 759 bp deletion in the γ 34.5 gene.
6. Use of a mutant herpes simplex virus according to any of the preceding claims as an anti-brain tumour agent.

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7. Use of a mutant herpes simplex virus according to claim 6 against primary tumours originating within the brain and nervous system.

8. Use of a mutant herpes simplex virus according to claim 6 against metastatic tumours, in particular against metastases of melanoma cancers.

9. Use of a mutant herpes simplex virus according to any of the preceding claims wherein the use is in a mammal, in particular in a human.

10. Use according to any of the preceding claims wherein the mutant herpes simplex virus is mutant 1716.

11. Use as an anti-cancer agent of a mutant herpes simplex virus type HSV-1 wherein the mutant virus comprises a modification in the γ 34.5 gene in the long repeat region (R_L) such that the γ 34.5 gene is non-functional; the anti-cancer use being in respect of cancer of the central nervous system including the brain, the cancer being a secondary metastatic tumour.

12. Use of a mutant herpes simplex virus according to any preceding claim in the manufacture of a medicament for the treatment of cancer in mammals, in particular in humans.

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13. Use of a mutant herpes simplex virus according to claim 12 in the manufacture of a medicament for the treatment of brain tumours in mammals, in particular in humans.

14. Use of a mutant herpes simplex virus according to claim 12 or claim 13 in the manufacture of a medicament for the treatment of primary tumours originating within the brain and/or nervous system.

15. Use of a mutant herpes simplex virus according to any of claims 12 to 14 in the manufacture of a medicament for the treatment of metastatic tumours, in particular against metastases of melanoma cancers.

16. A method of treating cancer in mammals, in particular in humans by administering a pharmaceutical formulation comprising a mutant herpes simplex virus type HSV-1 wherein the mutant virus is a mutant strain 17 virus and comprises a modification in the $\gamma 34.5$ gene in the long repeat region (R_L) such that the $\gamma 34.5$ gene is non-functional.

17. A method of treating cancer in mammals, in particular in humans according to claim 16 by administering a pharmaceutical formulation directly into the tumour.

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RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TREATMENT OF CANCER USING HSV MUTANT

the specification of which (check applicable box(es)):

☐ is attached hereto
☐ was filed on 28 January 1997 as U.S. Application Serial No. _____ (Atty Dkt. No. 117-231).
☒ was filed as PCT international application No. PCT/GB95/01791 on 28 July 1995
 and (if applicable to U.S. or PCT application) was amended on 16 August 1996

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Prior Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
9415320.2	United Kingdom	29 July 1994

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	Day/Month/Year Filed

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s):

Application Serial No.	Day/Month/Year Filed	Status: patented, pending, abandoned
PCT/GB95/01791	28 July 1995	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Paul J. Henon, 33626; Jeffrey H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr., 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; William J. Griffin, 31260; Robert A. Molan, 29834.

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Post Office Address: 2409 Manning Street, Philadelphia, Pennsylvania, United States of America
(Zip Code) 19103

FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet with same information and signature and date for each.

2025-04-04 09:40:00

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TREATMENT OF CANCER USING HSV MUTANT

the specification of which (check applicable box(s)):

☐ is attached hereto
☐ was filed on 28 January 1997 as U.S. Application Serial No. (Atty Dkt. No. 117-231)
☒ was filed as PCT international application No. PCT/GB95/01791 on 28 July 1995
and (if applicable to U.S. or PCT application) was amended on 16 August 1996

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Prior Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
<u>9415320.2</u>	<u>United Kingdom</u>	<u>29 July 1994</u>

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	Day/Month/Year Filed
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I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s):

Application Serial No.	Day/Month/Year Filed	Status: patented, pending, abandoned
<u>PCT/GB95/01791</u>	<u>28 July 1995</u>	<u>Pending</u>

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Beshar, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Paul J. Henon, 33626; Jeffrey H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr., 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; William J. Griffin, 31260; Robert A. Molan, 29834.

1.	Inventor's Signature: <u>[Signature]</u>	Date: _____			
	Inventor: <u>Alasdair</u> <u>Roderick</u> <u>MACLEAN</u>				
	(first) <u>MI</u> (last)				<u>British</u>
	Residence: (city) <u>Glasgow</u>	(state/country) <u>United Kingdom</u>			
	Post Office Address: <u>36 Colwood Place, Glasgow, United Kingdom</u>				
	(Zip Code) <u>G53 7YB</u>				
2.	Inventor's Signature: <u>[Signature]</u>	Date: _____			
	Inventor: <u>Susanne</u> <u>Moir</u> <u>BROWN</u>				
	(first) <u>MI</u> (last)				<u>British</u>
	Residence: (city) <u>Glasgow</u>	(state/country) <u>United Kingdom</u>			
	Post Office Address: <u>5 Bellshaugh Road, Glasgow, United Kingdom</u>				
	(Zip Code) <u>G12 0SN</u>				
3.	Inventor's Signature: <u>[Signature]</u>	Date: <u>4/11/97</u>			
	Inventor: <u>Nigel</u> <u>William</u> <u>FRASER</u>				
	(first) <u>MI</u> (last)				<u>British</u>
	Residence: (city) <u>Philadelphia</u>	(state/country) <u>Pennsylvania, United States of America</u>			
	Post Office Address: <u>511 South 46th Street, Philadelphia, Pennsylvania, United States of America</u>				
	(Zip Code) <u>19143</u>				

FOR ADDITIONAL INVENTORS, check box ☒ and attach sheet with same information and signature and date for each.

FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet with same information and signature and date for each.

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08/776350



UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: ASSISTANT COMMISSIONER FOR PATENTS
Box PCT
Washington, D.C. 20231

#2

U.S. APPLICATION NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
08/776,350	MACLEAN	A 117-231
INTERNATIONAL APPLICATION NO.		
PCT/GB95/01791		
I.A. FILING DATE		PRIORITY DATE
07/28/95		07/29/94

5621
NIXON & VANDERHYE
1100 NORTH GLEBE ROAD
8TH FLOOR
ARLINGTON, VA 22201

DATE MAILED: 03/19/97

NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

1. The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as

- ☐ a Designated Office (37 CFR 1.494),
☒ an Elected Office (37 CFR 1.495):

☒ U.S. Basic National Fee.

☒ Copy of the international application in:

- ☐ a non-English language.
☒ English.

☐ Translation of the international application into English.

☐ Oath or Declaration of inventors(s) for DO/EO/US.

☐ Copy of Article 19 amendments.

☐ Translation of Article 19 amendments into English.

☒ The International Preliminary Examination Report in English and its Annexes, if any.

☐ Translation of Annexes to the International Preliminary Examination Report into English.

☒ Preliminary amendment(s) filed 28 JAN 97 and

☒ Information Disclosure Statement(s) filed 28 JAN 97 and

☐ Assignment document.

☐ Power of Attorney and/or Change of Address.

☐ Substitute specification filed

☐ Verified Statement Claiming Small Entity Status.

☒ Priority Document.

☒ Copy of the International Search Report ☒ and copies of the references cited therein.

☐ Other:

2. The following items MUST be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- ☐ a. Translation of the application into English. Note a processing fee will be required if submitted later than the appropriate 20 or 30 months from the priority date.

☐ The current translation is defective for the reasons indicated on the attached Notice of Defective Translation.

- ☐ b. Processing fee for providing the translation of the application and/or the Annexes later than the appropriate 20 or 30 months from the priority date (37 CFR 1.492(f)).

☒ c. Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date.

☐ The current oath or declaration does not comply with 37 CFR 1.497(a) and (b) for the reasons indicated on the attached PCT/DO/EO/917.

- ☐ d. Surcharge for providing the oath or declaration later than the appropriate 20 or 30 months from the priority date (37 CFR 1.492(e)).

3. Additional claim fees of \$ _____ as a ☐ large entity ☐ small entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due. See attached PTO-875.

ALL OF THE ITEMS SET FORTH IN 2(a)-2(d) AND 3 ABOVE MUST BE SUBMITTED WITHIN ONE MONTH FROM THE DATE OF THIS NOTICE OR BY ☐ 21 OR ☒ 31 MONTHS FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

4. Translation of the Annexes MUST be submitted no later than the time period set above or the annexes will be cancelled. Note processing fee will be required if submitted later than 30 months from the priority date.

5. ☐ The Article 19 amendments are cancelled since a translation was not provided by the appropriate 20 (37 CFR 1.494(d)) or 30 (37 CFR 1.495(d)) months from the priority date.

Applicant is reminded that any communication to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above. (37 CFR 1.5)

A copy of this notice MUST be returned with this response.

Enclosed: ☐ PCT/DO/EO/917

☐ Notice of Defective Translation

☐ PTO-875

Vonda M. Wallace
Paralegal Specialist

FORM PCT/DO/EO/905 (September 1996)

Telephone: (703)

305-3736

08/776350-041807

08/776350



UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: ASSISTANT COMMISSIONER FOR PATENTS
Washington, D.C. 20231

#4

U.S. APPLICATION NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
08/776,350	MACLEAN	A 117-231

NIXON & VANDERHYE
1100 NORTH GLEBE ROAD
8TH FLOOR
ARLINGTON, VA 22201

5621

INTERNATIONAL APPLICATION NO.
PCT/GB95/01791

I.A. FILING DATE	PRIORITY DATE
07/28/95	07/29/94

DATE MAILED: 04/25/97

**NOTIFICATION OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C. 371
AND 37 CFR 1.494 OR 1.495**

1. The applicant is hereby advised that the United States Patent and Trademark Office in its capacity as ☐ a Designated Office (37 CFR 1.494), ☒ an Elected Office (37 CFR 1.495), has determined that the above identified international application has met the requirements of 35 U.S.C. 371, and is **ACCEPTED** for national patentability examination in the United States Patent and Trademark Office.

2. The United States Application Number assigned to the application is shown above and the relevant dates are:

18 APR 1997

35 U.S.C. 102(e) DATE

18 APR 1997DATE OF RECEIPT OF
35 U.S.C. 371 REQUIREMENTS

A Filing Receipt (PTO-103X) will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE "FILING DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371(C) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN ABOVE.** The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363). Once the Filing Receipt has been received, send all correspondence to the Group Art Unit designated thereon.

3. ☒ A request for immediate examination under 35 U.S.C. 371(f) was received on **28 JAN 1997** and the application will be examined in turn.

4. The following items have been received:

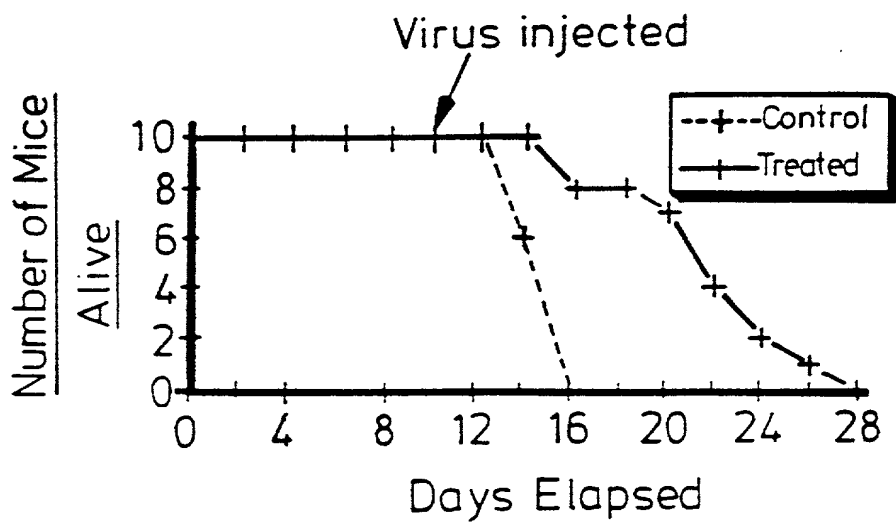
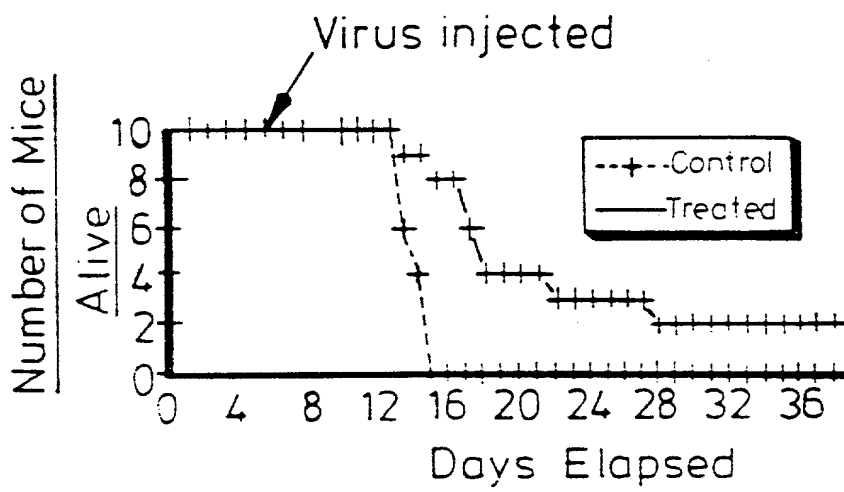
- ☒ U.S. Basic National Fee.
- ☒ Copy of the international application in:
 - ☐ a non-English language.
 - ☒ English.
- ☐ Translation of the international application into English.
- ☒ Oath or Declaration of inventor(s) for DO/EO/US.
- ☐ Copy of Article 19 amendments. ☐ Translation of Article 19 amendments into English.
- ☐ The Article 19 amendments ☐ have ☐ have not been entered.
- ☒ The International Preliminary Examination Report in English and its Annexes, if any.
- ☐ Translation of Annexes to the International Preliminary Examination Report into English.
- ☐ The Annexes ☒ have ☐ have not been entered.
- ☒ Preliminary amendment(s) filed **28 JAN 1997** and _____.
- ☒ Information Disclosure Statement(s) filed **28 JAN 1997** and _____.
- ☐ Assignment document.
- ☐ Power of Attorney and/or Change of Address.
- ☐ Substitute specification filed _____.
- ☐ Verified Statement Claiming Small Entity Status.
- ☒ Priority Document.
- ☒ Copy of the International Search Report ☒ and copies of the references cited therein.
- ☐ Other:

Applicant is reminded that any communication to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above. (37 CFR 1.5)

Vonda M. Wallace
Paralegal Specialist

V.W.

Telephone: (703) **305-3736**

1-9FIG. 1aFIG. 1b

2-9

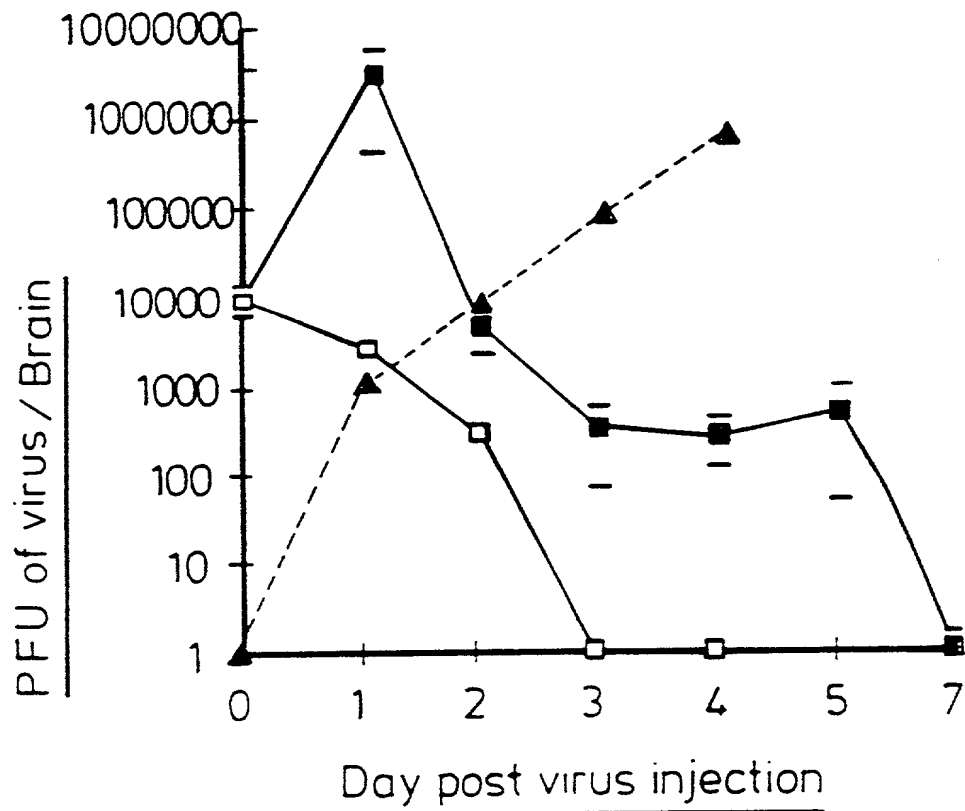


FIG. 2

3-9

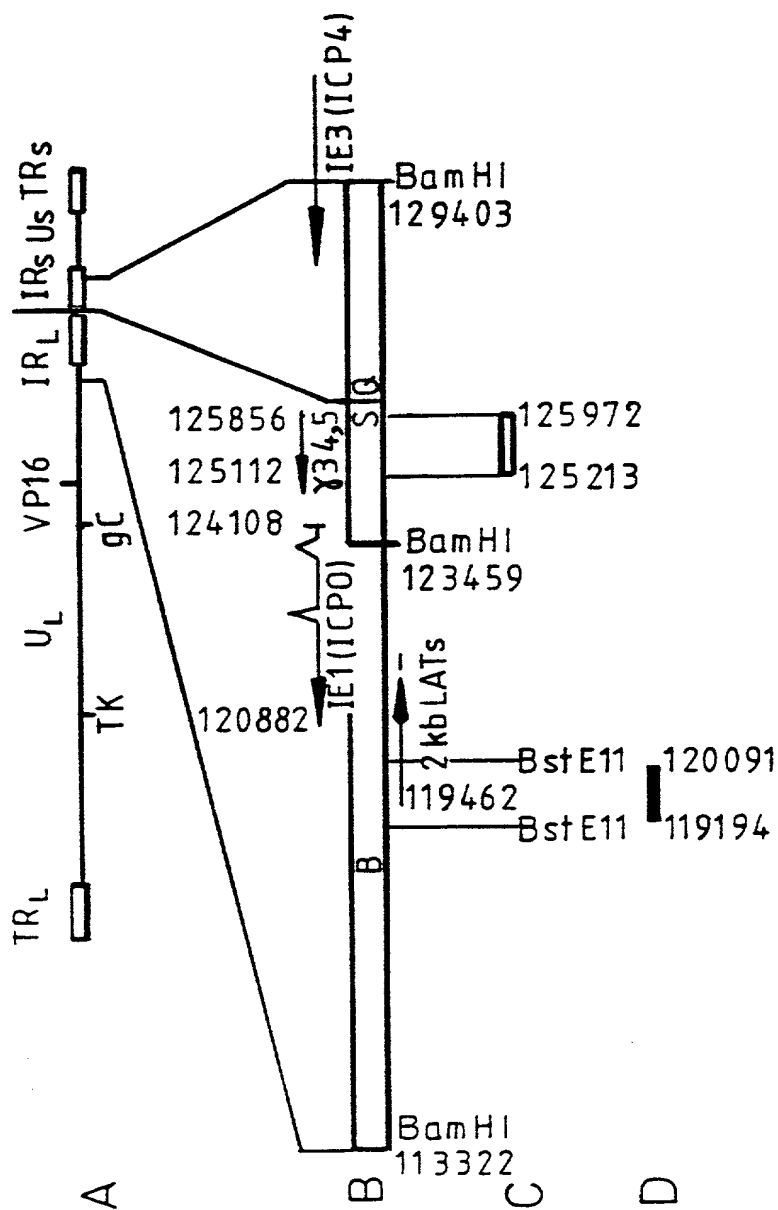
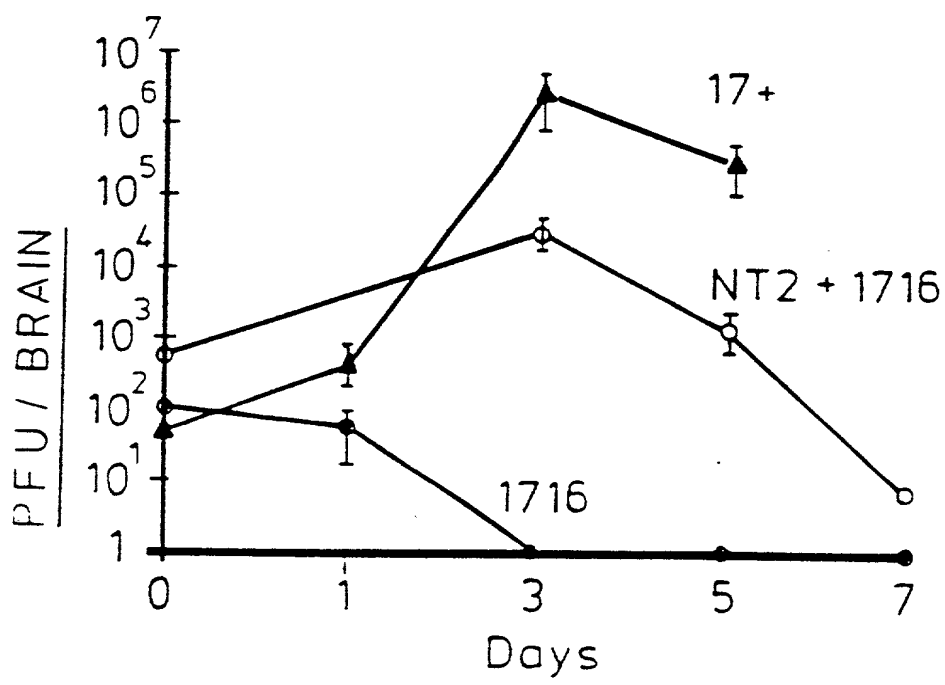
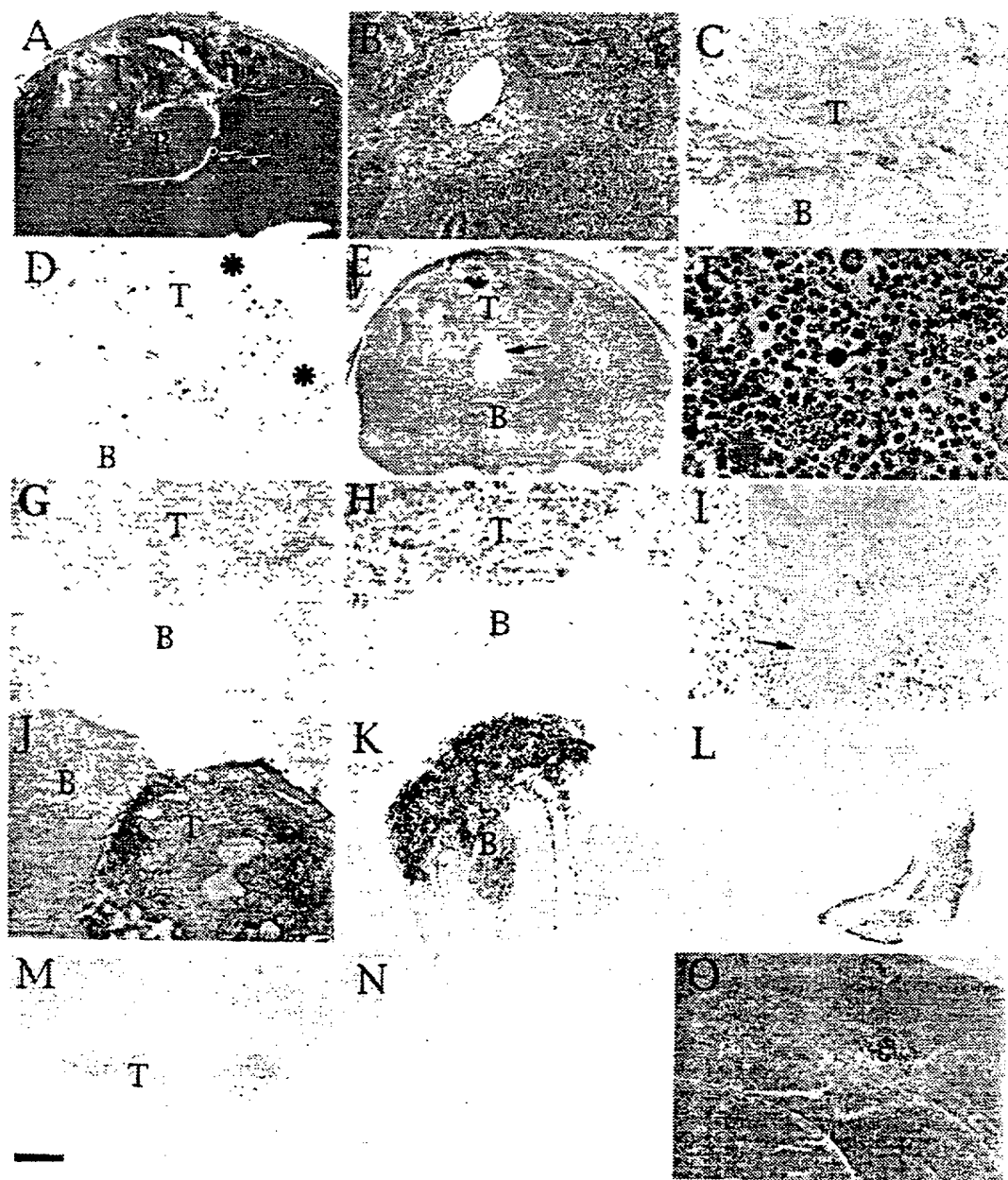


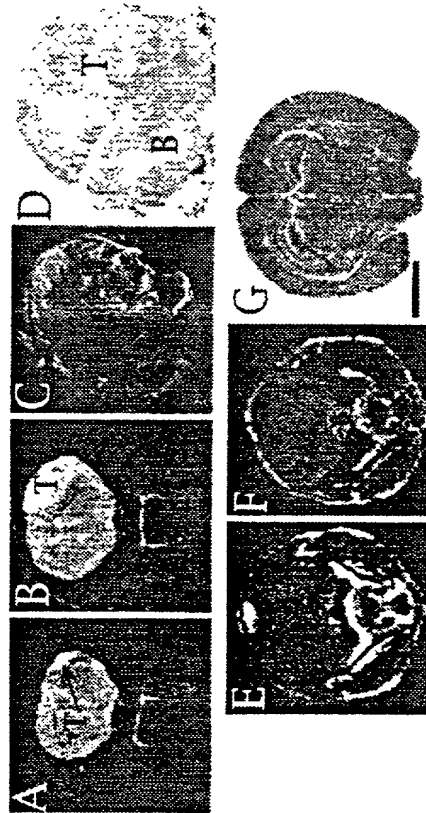
FIG. 3

4-9FIG. 4

5-9

FIG. 5

6-9

FIG. 6

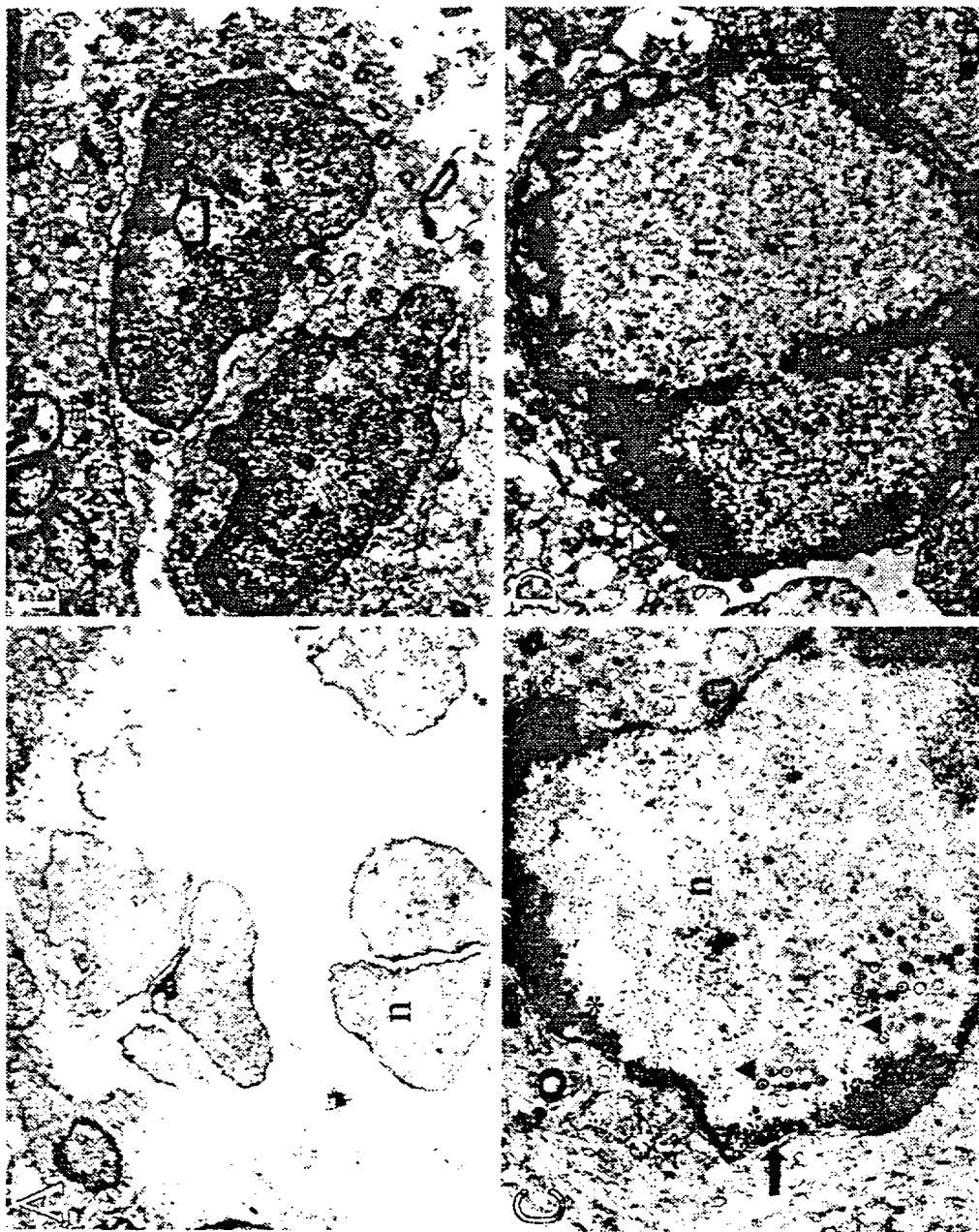
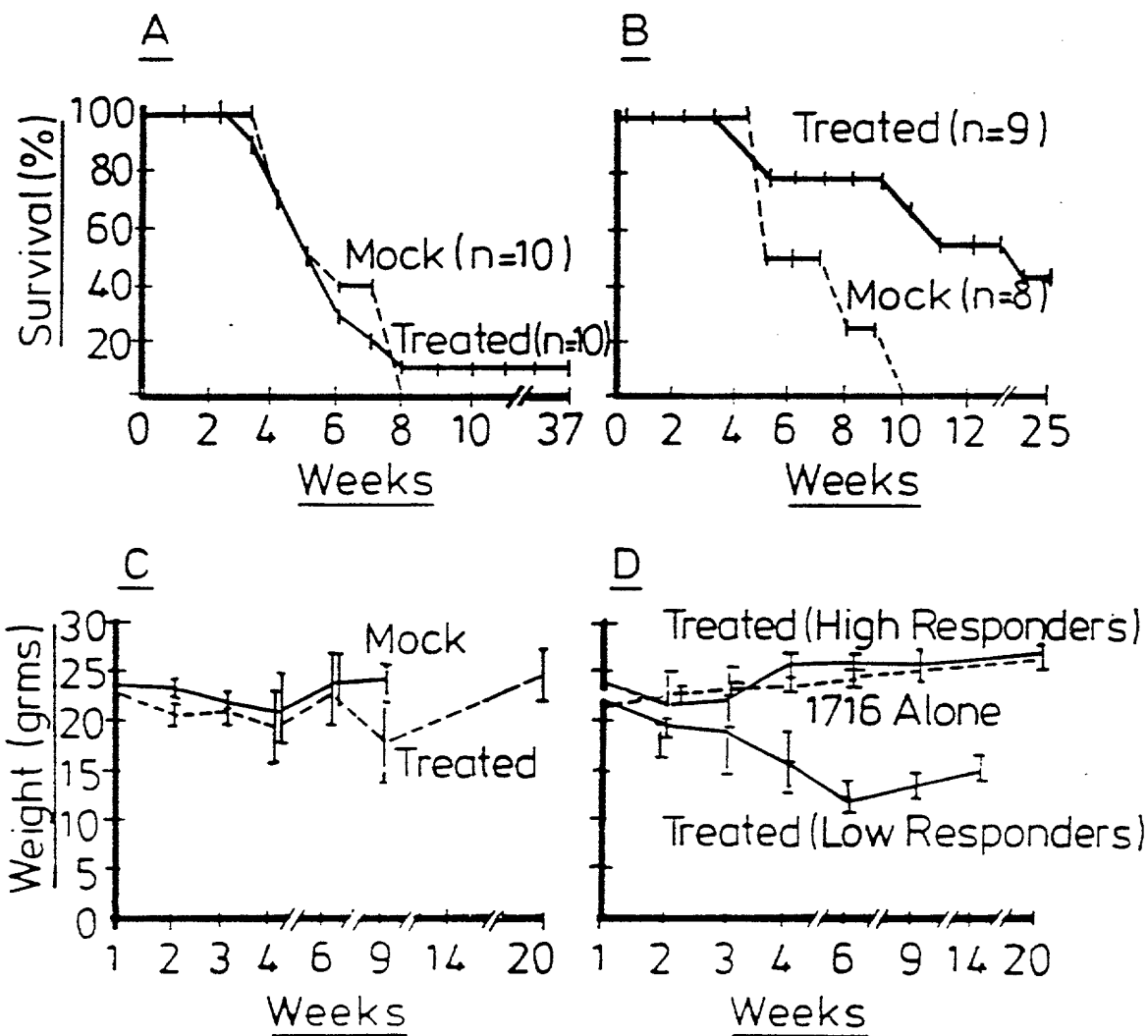


FIG. 7

8-9FIG. 8

9-9

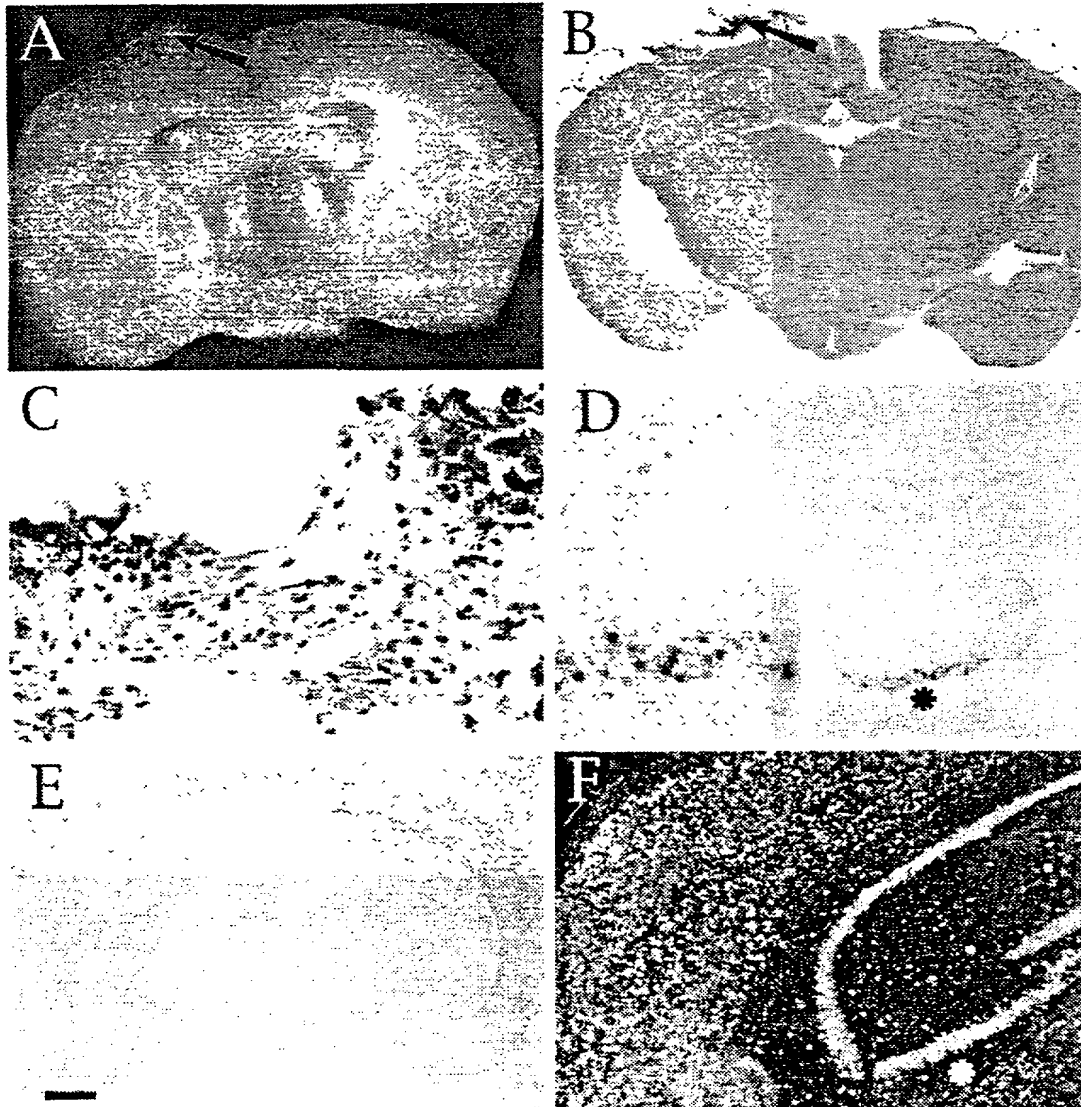


FIG. 9